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13. ABSTRACT (*Maximum 200 words*)

Surprisingly little is known about the mechanisms of bacterial colonization of surfaces in the marine environment, especially biological tissues. We are examining the process by which the bioluminescent bacterium Vibrio fischeri colonizes the nascent light organ of newly hatched juveniles of the squid Euprymna scolopes. This association has a number of advantages for such studies, in particular the high species specificity of the symbiosis, its monspecific nature, the ease of handling of the host, and the ability to genetically manipulate the bacterium. Using this system we have developed a number of genetic approaches to produce mutant strains of V. fischeri, and examined their colonization capacity as a means to understand the specificity of the association and its underlying biochemistry. In addition, we have used the natural diversity of light organ symbioses to examine the process of competition between closely related strains for colonization sites in the light organ. Final, we have discovered that the bacteria enter a "viable but nonculturable" state after they leave the host, but can be recovered from this dormancy during the infection of a subsequent host.

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FINAL REPORT

Grant #: N00014-93-I-0846

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GRANT TITLE: Bioluminescence and symbiosis

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OBJECTIVES:

(i) examine symbiotic competitive dominance in genetically distinct *Vibrio fischeri*;
(ii) identify nonculturable but viable and symbiotically active *V. fischeri* cells in seawater;
(iii) develop molecular genetic tools and approaches in symbiotic strains of *V. fischeri*;
(iv) identify the "microecological" characteristics of the environment of the symbiotic squid light organ crypts using defined mutant strains *V. fischeri*; and,
(v) determine the identity of symbiotic bacteria from light organs of *Sepiola* spp.

APPROACH:

A squid colonization assay was developed using aposymbiotic juveniles of the bioluminescent squid *Euprymna scolopes*. Specific gene probes were used to detect and quantify the presence of viable, nonculturable *V. fischeri* cells in Hawaiian seawater. Molecular genetic tools (conjugation, electroporation and transduction) were developed to manipulate and identify genes in *V. fischeri*. Symbiotic competency was tested for *V. fischeri* strains with defined mutations. Symbiotic bacteria from *Sepiola* spp were identified by cloning and sequencing of the DNA encoding their 16S rRNA gene, and their relative symbiotic competence was determined.

ACCOMPLISHMENTS:

We have now identified a total of 6 genetic loci (including both symbiosis-related genes, and non-symbiotic genes) in *V. fischeri* that have been used as population-specific markers in RFLP analyses of relatedness of different strains of this species. The results suggest that the genetic background of strains of *V. fischeri* can be better predicted by their ecological niche (symbiotic or freeliving) than there geographical occurrence. More detailed sequencing studies of the *gap* gene from different symbiotic strains of *V. fischeri* have provided strong evidence for coevolution between these bacteria and their host species.

We have collected symbiotic *V. fischeri* strains from the light organs of a third species of squid, *E. tasmanica*, from various locations in Australia. In addition we have isolated the symbionts of three species of *Sepiola* from the Mediterranean and found them to exist as a mixed culture of *V. logei* and *V. fischeri*.

Competitive dominance between the different strains of *V. fischeri* has been described during the initiation and maintenance of the light organ symbiosis. The dominance expresses itself after the first 24 h, and appears not to correlate to a differential ability to accumulate free iron,; however, preliminary studies with GFP-labeled cells suggest that dominant strains adhere more effectively to the host cells lining the light organ.

We have also identified a number of characteristics of the "microecology" of the interaction between the symbionts and the light organ crypt environment of the newly hatched squid. As part of these efforts we have developed a method for obtaining crypt contents directly from the animal, which has made analysis of the symbionts and their milieu possible.

We developed conjugation between *E. coli* and symbiotic *V. fischeri* as the basis of two distinct methods for performing transposon mutagenesis in *V. fischeri* cells. We used these procedures to produce mutant strains that are deficient in either the extent of the colonization, or in the persistence of the symbiotic colonization (see next paragraphs). Conditions for electroporation in *V. fischeri* were devised to introduce plasmid DNA isolated either from *V. fischeri*, or from strains of *E. coli* that are defective for the Dam methylase function, and have obtained approximately 10^4 electrotransformants per μg of plasmid DNA. This technique has allowed the construction of several gene replacement mutations. A third technique for DNA transfer in *V. fischeri* that we have developed is that of generalized transduction, a vital tool with which we can move genetic markers from one *V. fischeri* strain to another. We showed that phage rp1 infects wild-type, symbiotic isolates from *E. scolopes*, and have optimized the conditions for high frequency transduction in these strains.

We showed that light organ symbionts from *E. scolopes* produce a siderophore as indicated by the colorimetric CAS agar halo reaction. This fact presented the possibility of determining whether the production of a siderophore was essential for successful colonization of this host. A miniTn*5*Cm mutant (designated SP301) produced practically no halo on CAS indicator agar, and had a severely depressed growth rate and yield in an iron-limited minimal medium, conditions that were reversible by the addition of $FeCl_3$. Supernatant from a culture of the parent strain, which contained siderophore, did not enhance growth of the mutant. Thus, the mutation affected not only siderophore production, but the synthesis of the siderophore receptor as well. The site of the insertion has been cloned and sequenced, and it appeared to be *glnD*, a gene involved in nitrogen regulation. Phenotypic and genetic complementation studies have verified that it is *glnD*. Taken together, these results suggest that this insertion affects the expression of several genes, including those regulating both siderophore and nitrogen metabolism.

Nine transposon mutants of *V. fischeri* that were each auxotrophic for a different amino acid were retained the ability to colonize the crypts of juvenile squid, albeit to levels of between 2% and 93% that of wild type. Therefore, the crypt environment must provide sufficient levels of these nutrients to allow the proliferation of *V. fischeri* cells. We have released the contents of the crypts of adult animals, and separated the symbiotic cells from the extracellular crypt matrix fluid in which they reside. Examination of this released material has revealed that 60% of the total amino acids present were in either an oligomeric (peptide) or polymeric (protein) form. In fact, if this material is entirely available to the growing bacterial population, it could theoretically provide for the total synthesis of all the symbiont cell protein. Thus, the symbionts themselves are unlikely to be the source of a significant amount of this material.

CONCLUSIONS: (It is not possible to describe all the conclusions from this period of support in the space available, but the following are representative.)

(i) The discovery of viable nonculturable *V. fischeri* cells changes the way we view the initiation of light organ symbiosis and the ecology of the bacterium and its host. Of even broader interest, it suggests that at least a portion of the 99.9% of bacteria in seawater that can not be cultured may not be unknown or new species, but in fact be well-known, typically culturable bacteria that are awaiting a specific environmental cue that signals these cells to grow;

(ii) The inability of motility mutants to initiate an infection indicates the need to swim through a viscous barrier in the crypts. The ability of siderophore and auxotrophic mutants to initiate but not persist normally in the light organ reveals important aspects of the host-produced nutritional conditions of the crypts;

(iii) The speciation of sepiolid squids has occurred coordinately with the speciation of symbiotic luminous bacteria, providing one of the best examples of coevolution between marine species;

(iv) The predominant symbiotic bacteria from the light organs of three species of *Sepiola*, the Mediterranean relatives of *Euprymna scolopes*, have been identified as *V. logei*. This species coexists in the light organs with cells of *V. fischeri* which it appears to dominate only under

seasonally cooler water conditions. This is the first report of *V. logei* as a light organ symbiont, and the first report of a mixed-species colonization of a symbiotic bioluminescent organ.

(v) Symbiotic competence (in juveniles of *E. scolopes*) differed among strains of *V. fischeri* isolates from six host species of *Euprymna* and *Sepiola*. The order of extent of colonization effectiveness was: *scolopes* > *morsei* >> *tasmanica* >> *Sepiola* species;

SIGNIFICANCE:

The results of this work address four important questions in marine symbioses:

(ii) Is there a significant population of typically culturable *V. fischeri* cells in seawater that have entered a non-culturable state from which they can be recovered only through an association with a specific animal tissue?

(ii) What are the genetic mechanisms underlying the symbiotic competence of luminous bacteria in the development of an association with their host?

(iii) What is the mechanistic basis underlying the symbiotic dominance of one subclass of marine bacteria in the development of an association with its host?

(iv) How have these bacterial symbiotic competence factors evolved during host speciation?

PATENT INFORMATION: None

AWARD INFORMATION:

(i) E.G. Ruby (PI): Promoted to Professor, USC, and Appointed Senior Researcher, UH.

(ii) K-H. Lee (graduate student): 1994 ASM Sarber Award as the top graduate student researcher in microbial ecology (there were 5 Sarber awards given at this 10,000 person meeting, only one of which was in ecology).

(iii) P. Fidopiastis (graduate student): 1997 ASM Travel Award.

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